

Case 518, 003

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
24 December 2003 (24.12.2003)

PCT

(10) International Publication Number
WO 03/105862 A1

(51) International Patent Classification⁷: A61K 31/70, 31/505, C07H 19/16, C07D 211/72, C07F 9/22, C07C 229/00, 241/00

Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(21) International Application Number: PCT/US03/18716

Declarations under Rule 4.17:

— as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, U4, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)

— as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, U4, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)

— of inventorship (Rule 4.17(iv)) for US only

Published:

— with international search report
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/105862 A1

(54) Title: IN-VIVO ENERGY DEPLETING STRATEGIES FOR KILLING DRUG-RESISTANT CANCER CELLS

(57) Abstract: This invention also provides a method for treating a cancer subject comprising administering to the subject a combination of ATP-depleting agents at concentrations which deplete the ATP level to, or close to, at least 15 % of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a methylthioadenosine phosphorylase inhibitor or an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, wherein said composition produces a substantially better effect than a composition without at least one of the ATP-depleting agents: a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor and an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside.

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**IN-VIVO ENERGY DEPLETING STRATEGIES FOR KILLING
DRUG-RESISTANT CANCER CELLS**

This application is a continuation-in-part of U.S. Serial No. 5 10/172,346, Filed 13 June 2002, the content of which is hereby incorporated into this application by reference.

The invention disclosed herein was made with government support under National Cancer Institute RAID Grant Application 10 #153. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various references are referred to. Disclosures of these publications in their entireties are 15 hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION AND CANCER RELEVANCE

20 Drug resistance is the central problem of cancer chemotherapy. Clinically effective combination chemotherapy can cause impressive objective tumor response rates, including complete tumor regressions, but some cancer cells are of lesser 25 sensitivity to the agent (i.e., are drug-resistant), are only damaged, recover, and re-grow. The delayed tumor recurrence yields only a short remission period with little improvement in survival time.

30 There are many mechanisms of drug resistance. Multiple independent mechanisms of drug resistance may coexist in a population of tumor cells as well as in the same cancer cells, as they arise from multiple genetic changes in single cell clones, and are part of the heterogeneity of the neoplastic 35 process. Mechanisms of drug resistance have been largely identified, and this knowledge has suggested many specific

approaches to overcoming one or another type of clinical drug resistance, but these attempts have failed as they also potentiate drug toxicity towards normal tissues.

5 The therapeutic research strategy for several decades has been that the administration of multiple drugs with different properties and mechanisms of action at optimal doses and intervals should result in cells resistant to one class of drug being killed by another drug in the regimen. However, the
10 extensive, clinical data over these decades has evidenced only a minor impact on treatment outcome along with troublesome and serious toxic side effects (e.g., emesis, diarrhea, alopecia, asthenia, fatigue, myelosuppression, febrile neutropenia requiring hospitalization, neurosensory and neuromotor
15 disturbances, arthralgias and myalgias, heart failure, and treatment-related deaths). Despite the long dismal history of repeated failures to meaningfully improve survival rates by aggressive combination chemotherapy with non-cross-reacting drugs, hope is nevertheless expressed that the future will be
20 different with the new molecularly targeted agents.

However, no matter how many effective mechanistically-different anticancer agents there are, and no matter how superior their therapeutic index, cancer cell demise occurs by
25 only two cell death pathways (necrosis or apoptosis). If the two cell death mechanisms are attenuated by drug resistance mechanisms (e.g. p-glycoprotein and/or glutathione prevent intracellular drug levels reaching concentration levels sufficient to fully activate the necrosis pathway; caspase
30 deletions and endogenous caspase inhibitors prevent completion of apoptosis), these tumor cells are only sublethally injured, recover, and proliferate to kill the patient. The history of the results of these clinical trials is therapeutic equivalence between different combination chemotherapy

"doublets" and "triplets". The repeated failures of this approach to overcoming clinical drug resistance - i.e., the lack of clinically relevant differences in overall survival means only continuance of palliative treatment with decisions 5 tailored individually around such issues as differences in toxicity profiles, patients' age and performance status, and quality of life.

10 New agents, no matter a new molecular target or superior therapeutic index, can only kill cancer cells if there is completion of the cell death pathways through death's door. In drug-resistant cells, it is not the activation of their cell death pathways by clinically effective anticancer agents that 15 is at fault, but rather pathway completion to death of the cell. This reality suggests that continuance of this failed strategy utilizing only aggressive combination chemotherapy with non-cross-reacting drug-will likely result in -to quote 20 Yogi Berra- "déjà vu all over again".

25 The above facts suggest the development of a treatment (co-administered with the initiation of activity in the cell death pathways by anticancer agents) that complements and augments the agent-induced initiation of activity in cell death pathways to bring the pathway to completion; namely, to cell death. That treatment, focused on severe ATP depletion, has been developed and proven at the preclinical level, and is about to undergo validation by clinical trial with clinical supplies of the ATP-depleting regimen provided by the NCI RAID 30 grant mechanisms.

30 Heterogeneous neoplastic cell populations likely contain cancer cells of variable sensitivity to the anticancer agents. Less sensitive cells would not receive enough damage to reduce ATP to low levels sufficient to cause necrotic death. We

hypothesized that biochemical modulation to further depress ATP to lower lethal-inducing levels would kill these sublethally-injured cells, augment tumor regressions, and perhaps even yield some cures.

SUMMARY OF THE INVENTION

This invention provides a composition comprising a combination of ATP-depleting agents at concentrations which deplete the ATP level to at least 15% of normal in cancer cells, wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof.

10

The ATP level is depleted to at least 15% of normal in cancer cells. In some embodiments, it is substantially lower than 15%. For example, the level could be 5% of normal. In a further embodiment, the level is as low as 1%.

15

This invention also provides a composition comprising an effective amount of a combination of ATP-depleting agents at concentrations which deplete the ATP level to at least 15% of normal in cancer cells, and a pyrimidine antagonist, wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof.

25

This invention also provides a method for treating a cancer subject comprising administering to the subject a combination of ATP-depleting agents at concentrations which deplete the ATP level to, or close to, at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a

combination ther

This invention also provides a method for treating a cancer subject comprising administering to the subject a combination of ATP-depleting agents at concentrations which deplete the ATP level to, or close to, at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof, wherein said composition produces a substantially better effect than a composition without at least one of the following ATP-depleting agents: a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof.

This invention also provides a method for induction of cancer cell death comprising contacting said cancer cell with a combination of ATP-depleting agents at concentrations which deplete the ATP level to at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof.

This invention also provides a method for induction of cancer cell death comprising contacting said cancer cell with a combination of ATP-depleting agents at concentrations which deplete the ATP level to at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a methylthioadenosine

phosphorylase inhibitor, an inhibitor of Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof, wherein said composition produces a substantially better effect than a composition without at least one of the following ATP-depleting agents: a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof.

This invention provides a method for treating a cancer subject, and for the induction of cancer cell death, comprising administering to the subject a combination of ATP-depleting agents, a pyrimidine antagonist, and anticancer agent to which the treated cancer is sensitive, at concentrations which together collectively deplete the ATP levels to at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof.

This invention provides a method for treating a cancer subject, and for the induction of cancer cell death, comprising administering to the subject a combination of ATP-depleting agents, a pyrimidine antagonist, and anticancer agent to which the treated cancer is sensitive, at concentrations which together collectively deplete the ATP levels to at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof,

wherein said composition produces an effect which is substantially better than a composition without at least one of the following ATP-depleting agents: a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof.

DETAILED DESCRIPTION OF THE INVENTION

Patent Cooperation Treaty (PCT) Application PCT/US01/46886 (International Publication Number WO 02/4720A1) discloses treatment of cancer by reduction of intracellular energy and pyrimidine. Specifically, PCT/US01/46886 highlighted the importance of depletion of ATP in cancer therapy. The disclosure herein provides improvements over this PCT application.

10 This invention provides a composition comprising a combination of ATP-depleting agents at concentrations which deplete the ATP level to at least 15% of normal in cancer cells, wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof.

20 The ATP level is depleted to at least 15% of normal in cancer cells. In some embodiments, it is substantially lower than 15%. For an example, the level could be 5 to 10 % of normal. In another instance, the level is 1 to 4 %. In a further embodiment, the level is as low as 1%.

25 This invention also provides a composition comprising an effective amount of a combination of ATP-depleting agents at concentrations which deplete the ATP level to at least 15% of normal in cancer cells, wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof.

5 This invention also provides the above compositions, wherein said compositions produce a substantially better effect than a composition without at least one of the following ATP depleting agents: a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof.

10 As used herein, substantially better means that the composition could deplete the intracellular energy level at least 5% better. In another embodiment, the composition is 5% to 100% better. In a separate embodiment, it is 10% to 100% better. In a further embodiment, it is 15% to 100% better.

15 In a still further embodiment, the composition is 20% to 100% better. In a further embodiment, it is 25% to 100% better. In another embodiment, it is 30% to 100% better. In a separate embodiment, it is 35% to 100% better. In another embodiment, it is 40% to 100% better. In a further embodiment, it is 45% to 100% better. In another embodiment, it is 50% to 100% better. In a still further embodiment, it is 55% to 100% better. In a separate embodiment, it is 60% to 100% better. In a still separate embodiment, it is 65% to 100% better. In another embodiment, it is 70% to 100% better. In a separate embodiment, it is 75% to 100% better. In another embodiment, it is 80% to 100% better. In a separate embodiment, it is 85% to 100% better. In a further embodiment, it is 90% to 100% better. Finally, the composition may be 95% to 100% better.

20

25

30 To achieve "substantially better than the composition without", it is not necessary that the depleting agents used be more than one agent. In fact, a single agent may be capable of performing such better effect.

5 The above compositions may further comprise a pyrimidine depleting agent. Further, the above compositions may comprise an anticancer agent to which the cancer is sensitive. In a still further embodiment, the anticancer agent is at approximately half the maximum tolerated dose.

10 These agents stated hereinabove may be a single agent but with more than one function. For example, an ATP-depleting agent may also be an anticancer agent.

15 The ATP-depleting agents include but are not limited to 6-methylmercaptopurine riboside (MMPR), 6-Aminonicotinamide (6-AN) or alanosine (AL).

20 The above compositions may further comprise N(phosphonacetyl)-L-aspartic acid (PALA). In a separate embodiment of the compositions, it comprises 3-bromopyruvic acid.

25 The above composition further comprises dehydroepiandrosterone (DHEA), oxythiamine (OT) or in combination thereof.

In an embodiment, the composition further comprises 6-Aminonicotinamide (6-AN).

30 The above composition may further comprise a cytokine. In an embodiment, the cytokine is G-CSF.

This invention also provides a pharmaceutical composition comprising the above composition and a pharmaceutically acceptable carrier.

For the purposes of this invention, "pharmaceutically acceptable carriers" mean any of the standard pharmaceutical

carriers. Examples of suitable carriers are well known in the art and may include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution and various wetting agents. Other carriers may 5 include additives used in tablets, granules and capsules, etc. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable 10 fats or oils, gum, glycols or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are 15 formulated by well-known conventional methods.

This invention also provides the combination of ATP-depleting 20 agents and the anticancer agent to treat drug-resistant cancer cells. In an embodiment, the dose of the anticancer agent is 25 at approximately half of the maximum tolerated dose.

This invention also provides a method for treating a cancer in 30 a subject comprising administering to the subject a combination of ATP-depleting agents at concentrations which deplete the ATP level to, or close to, at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside or 35 combination thereof.

This invention also provides a method for treating a cancer 40 subject comprising administering to the subject a combination of ATP-depleting agents at concentrations which deplete the ATP level to, or close to, at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a 45

mitochondrial ATP-inhibitor, a glycolytic inhibitor, methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof, and said composition 5 produces a substantially better effect than a composition without at least one inhibitor of the following ATP-depleting agents: a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine 10 riboside or a combination thereof.

This invention also provides the above methods wherein the agents further comprise a pyrimidine-depleting agent.

15 The above methods may further comprise an anticancer agent. In another embodiment, the cancer is clinically sensitive to the employed anti-cancer agent.

20 In a separate embodiment, the anticancer agent is at approximately half of the maximum tolerated dose.

This invention also provides a method for induction of cancer cell death comprising contacting said cancer cell with a combination of ATP-depleting agents at concentrations which 25 deplete the ATP level to at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a 30 combination thereof.

This invention also provides a method for induction of cancer cell death comprising contacting said cancer cell with a

1 combination of ATP-depleting agents at concentrations which
deplete the ATP level to at least 15% of normal in cancer
cells wherein at least one of the ATP-depleting agents is a
mitochondrial ATP-inhibitor, a methylthioadenosine
5 phosphorylase inhibitor, an inhibitor of De Novo purine
synthesis other than 6-Methylmercaptopurine riboside, or a
combination thereof, wherein said composition produces a
substantially better effect than a composition without at
least one of the following ATP-depleting agents: a
10 mitochondrial ATP-inhibitor, a glycolytic inhibitor, a
methylthioadenosine phosphorylase inhibitor, an inhibitor of
De Novo purine synthesis other than 6-Methylmercaptopurine
riboside, or a combination thereof.

15 The above methods may comprise a pyrimidine-depleting agent.
In an embodiment, the above methods further comprise an
anticancer agent.

20 In an embodiment, the cancer is clinically sensitive to the
employed anticancer agent. In a further embodiment, the
anticancer agent is at approximately half of the maximum
tolerated dose.

25 This invention provides a method for treating a cancer
subject, and for the induction of cancer cell death,
comprising administering to the subject a combination of ATP-
depleting agents, a pyrimidine antagonist, and anticancer
agent to which the treated cancer is sensitive, at
concentrations which together collectively deplete the ATP
30 levels to at least 15% of normal in cancer cells wherein at
least one of the ATP-depleting agents is a mitochondrial ATP-
inhibitor, a glycolytic inhibitors, a methylthioadenosine
phosphorylase inhibitor, an inhibitor of De Novo purine

synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof.

This invention provides a method for treating a cancer subject, and for the induction of cancer cell death, comprising administering to the subject a combination of ATP-depleting agents, a pyrimidine antagonist, and anticancer agent to which the treated cancer is sensitive, at concentrations which together collectively deplete the ATP levels to at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof, wherein said composition produces an effect which is substantially better than a composition without at least one of the following ATP-depleting agents: a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof.

In an embodiment of the above methods, the anticancer agent is at approximately half of the maximum tolerated dose.

The ATP-depleting agents used in the above methods include but are not limited to 6-methylmercaptopurine riboside (MMPR), 6-Aminonicotinamide (6-AN) and alanosine (AL).

In an embodiment of the above methods, the methods further comprise N-(phosphonacetyl)-L-aspartic acid (PALA).

In an embodiment of the above methods, the method further

comprises 3-bromo-¹³C-¹³pyruvic acid.

In an embodiment of the above methods, the method further comprises N-(phosphonacetyl)-L-aspartic acid (PALA).

5

In an embodiment of the above methods, the method further comprise dehydroepiandrosterone (DHEA), oxythiamine (OT) or in combination thereof.

10 In an embodiment of the above methods, the method further comprises 6-Aminonicotinamide (6-AN).

15 In an embodiment of the above methods, the method further comprises a cytokine. The cytokine includes but is not limited to G-CSF.

This invention also provides a method for killing drug-resistant cancer cells comprising contacting the combination of ATP-depleting agents and the anticancer agent. In an embodiment, the dose of the anticancer agent is at 20 approximately half of the maximum tolerated dosage.

25 This invention provides a method for treating drug-resistant cancer cells comprising contact the said cancer with a combination of ATP-depleting agents and an anticancer agent.

In an embodiment the dose of said anticancer agent is at approximately half of the maximum tolerated dose.

30 This invention provides the above method, wherein the ATP level is depleted to at least 15% of normal in cancer cells and at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a

methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof.

5 This invention further provides the above described method wherein the ATP level is depleted to at least 15% of normal in cancer cells and at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of 10 De Novo purine synthesis other than 6-Methylmercaptopurine riboside, or a combination thereof, within and said 15 composition produces an effect which is substantially better than a composition without at least one of the ATP-depleting agents: a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor, an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside or a combination thereof.

Finally, this invention provides method for induction of 20 cancer cell death comprising contacting said cancer cell with an agent capable of inducing necrosis in cancer cells. In an embodiment the agent is an ATP-depleting agent. This method may include a pyrimidine-depleting regimen, or a combination thereof.

25 The invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative, and are not meant to limit the 30 invention as described herein, which is defined by the claims which follow thereafter.

Experimental Details

MAP

An ATP- reducing regimen, MAP--- an acronym for the combination of 6-methylmercaptopurine riboside, MMPR, + 6-aminonicotinamide, 6-AN, + N- (phosphonoacetyl) -L- aspartic acid, PALA) --- when co--administered with anticancer agents; e.g., Doxorubicin A (Adriamycin)--- enhances experimental tumor regressions in vivo via the necrosis cell death pathway (1). MMPR is an inhibitor of de novo purine biosynthesis that limits adenine supplies for ATP production. (2) 6-AN is an inhibitor of the generation of ATP in the glycolysis pathway (3-6). The cell-killing ATP threshold is 15% of basal levels, and lower (7-8). Since multiple biochemical pathways contribute to the synthesis, generation and maintenance of ATP, a combination of two ATP-depleting agents, MMPR + 6-AN, is co-administered with effective anticancer agents to concomitantly block several of the ATP- producing pathways to achieve the ATP- depleting objective of \leq 15% of normal. PALA, a de novo pyrimidine biosynthesis inhibitor, can, at low non-toxic dosages, selectively lower pyrimidine nucleotide levels in tumors (9). MMPR + 6-AN can markedly lower ATP levels to an average of 15% of normal in murine mammary cancers (1). In the presence of such severely depleted ATP levels the salvage pathway formation of pyrimidine di-and tri-phosphates can be inhibited at the kinase step. Biochemical analysis shows that MAP-treated tumors are severely depleted of two vitally needed metabolites: the UTP pools to 14% of normal (10), and ATP to 15% of normal (1).

Necrosis cell death ensues because intracellular ATP levels at 15% of basal or lower can not sustain cellular homeostasis (7-8, 11-12). Indeed, intracellular ATP levels determine cell fate by apoptosis or necrosis (11) and can effect a "switch" in the cell death mode between apoptosis and necrosis (12). There are many references that anticancer agents that usually

cause cell death by apoptosis, when blocked by exogenous or endogenous caspase inhibitors, or by manipulation (i.e., depletion) of energy metabolism, "switch" to necrosis cell death due to severe ATP depletion (references summarized in Table 1).

TABLE 1: EVIDENCE IN NON-DRUG RESISTANT CELLS THAT APOPTOTIC INDUCERS CAN BE MADE TO "SWITCH" TO THE NECROSIS CELL DEATH PATHWAY

No	Apoptotic Inducer	Cells	Apoptosis Blocker	Result	Reference
1	EGF	Pancreas	Caspase1-inhibitor	Apoptosis to non-apoptotic 'necrotic'-like deaths	FEBS Lett. 491:1 08-108, 2001
2	Staurosporin, VP-16, Act D	Jurkat Lymphoma T-cell	Inhibitor of caspases	Apoptosis to necrosis	Cell Death Differ. 5:298-306, 1998
3	TNF	L929 and HeLa cells	Inhibitor of caspases	Enhanced necrosis	J. Exp. Med. 187: 1477-1485, 1998
4	Staurosporin, CD95 stimulation	Human T cells	Pre-empted cells of ATP	Apoptosis to necrosis	Cell Death Differ. 4: 435-442, 1997
5	Fas mAb stimulation	Jurkat T cells, HeLa	ATP depletion	Apoptosis to necrosis	Cancer Res. 57: 1835-1840, 1997
6	Dexamethasone	B-lymphocytes	Inhibitor of caspases	Apoptosis to necrosis	FEBS Lett. 425: 266-270, 1998
7	Dexamethasone Etoposide	Thymocytes	Inhibitor of caspases	Apoptosis to necrosis	Oncogene 15: 1573-1581, 1997
8	Staurosporin, CD95 agonist	Human T cells	Per-empted cells of ATP	Apoptosis to necrosis	J. Exp. Med. 185: 1481-1486, 1997
9	Deoxycholic acid (activates Fas)	HCT 116 cells	Overexpression of bcl-2 or PKC	Apoptosis to necrosis	Cancer Lett. 152: 107-113, 2000
10	H ₂ O ₂	HN U937 cells	Increasing H ₂ O ₂ concentration	Apoptosis to necrosis	Exp. Cell Res 221:462-469, 1995
11	Bax	Jurkat cell	Inhibitor of caspases	Apoptosis to "non-apoptotic"	Proc Natl Acad Sci. 93: 14559,

				death	1996
12	Camptothecin	Leukemia U-937 cells	Inhibitor of caspases	Apoptosis to necrosis	Cancer Res. 59: 3565-3569, 1999
13	Anti-F25 Abs	L 929 cells	Inhibitor of caspases	Apoptosis to necrosis	J. Exp. Med. 188: 919-930, 1998
14	Anti-F25 Abs	Jurkat cells	Inhibitor of caspases	Apoptosis to necrosis	J. Cell Biol. 143:1353-1360, 1998
15	TNF- α	Mouse 3T3 fibroblasts	Inhibitor of caspases	Normally resistant; switch to necrosis	J. Virol. 74: 7470-7477, 2000

The reason is that anticancer agents usually damage DNA, either directly or indirectly, and thereby activate the two principal pathways of cell death, necrosis and apoptosis, simultaneously in the same cancer cell. Only one death pathway prevails in the cells, as determined by intracellular conditions. However, the two modes of cell death can occur in different cells simultaneously in the same tumor exposed to the same anticancer agent. One reason is that different drug concentrations reach different cancer cells; low concentrations induce apoptosis and high concentrations cause necrotic cell death (13). If the anticancer drug's intracellular concentration is high, the drug-DNA "hit" is then of sufficient magnitude to strongly activate poly (ADP-ribose) polymerase (PARP), and PARP rapidly destroys and depletes glycolytic NAD severely inhibiting the glycolytic production of ATP (1). The resulting severe depletion of ATP causes cell death by necrosis (1). And, although low concentrations of anticancer agents do not damage DNA sufficiently to cause the marked lowering of ATP that results in necrotic cell death, there is still a reduction of ATP. Hence, all effective anticancer agents always cause ATP reduction, and are considered part of our ATP-depleting regimen. An important part, because as "selective" agents for

5 cancer cells, [] effective anticancer agent [] preferentially reduce more ATP in cancer cells than normal cells. The agents thereby create a therapeutic opportunity for co-administration of biochemical modulators (i.e., the concomitant addition of an ATP-depleting regimen—e.g., MAP) to further reduce cancer cell ATP to the severely low levels (i.e., \leq 15% of normal) that kill, and the cells die a necrotic death.

10 MAP plus each of ten mechanistically different anticancer drugs at half-MTD (doxorubicin, paclitaxel, docetaxel, cisplatin, phenylalanine mustard, mitomycin C, cyclophosphamide, etoposide, 5-fluorouracil, and radiotherapy) has been demonstrated in vivo to be safe and to cause 15 significantly greater tumor-regressions than the MTD of each anticancer agent alone in hundreds of animals bearing advanced spontaneous and first generation murine breast cancer (references summarized in 1), as well as in murine leukemia and colon tumors. "Cures" (25%) were produced with MAP + 20 radiotherapy in tumor-bearing mice followed for over 380 days (14). The improved therapeutic results were all produced by MAP + each anticancer agent at half-MTD, strongly suggesting that a marked reduction of the toxic side effects of usual MTD clinical cancer chemotherapy will be obtained in future 25 clinical trials.

25 Spontaneous murine breast cancers have drug-resistant cancer cells as part of their heterogeneous neoplastic cell population just as spontaneously-arising human cancers do. The 30 fact that the number of tumor regressions induced by the combination of MAP+ anticancer agents were always significantly greater than the regressions produced by each anticancer agents alone at MTD suggested that the additional cell kills occurred in drug-resistant cancer cells. Drug-

resistant cancer cells are known to be frequently blocked in apoptosis, but not known to have blocks per se in the necrosis cell death pathway. It is likely that drug-resistant cells were killed in the spontaneous tumor because the ATP-depleting strategy is specifically targeted at the latter pathway. 5 However, to obtain a clearer focus of the effect of the ATP-depleting strategy plus an anticancer agent on drug-resistant cancer cells, treatment was done on multi-drug-resistant, overexpressed p-glycoprotein NCI-AR-Res (Adriamycin-resistant) 10 human mammary carcinoma xenografts (15). (See Table 2, below)

MAPAL

As previously noted, the ATP depletion induced in murine mammary tumors by (MMPR+ 6-AN) of MAP averaged 15% of normal, 15 the cytocidal threshold level of ATP shown to be insufficient to sustain cell viability (7-8). Hence, some individual tumor cells have ATP levels < 15% of normal, are killed, and the tumors regress. Other MAP-treated tumors have reduced ATP levels > 15% of normal and these tumors are only inhibited in 20 their growth (7). Obviously if the (MMPR+ 6-AN) induced ATP tumor level had averaged lower than 15% of normal, there would be more tumor cells killed, and more tumor regressions. A stronger ATP-depleting regimen was therefore sought. Alanosine (AL), an inhibitor of de novo adenosine monophosphate 25 synthesis (16), had been previously shown to add to MMPR's reduction of ATP (17). In the latter studies, MMPR alone lowered ATP levels to 49% of normal, and alanosine (AL) alone to 63% of normal, but in combination they decreased ATP to 34% of normal. This striking synergistic reduction of ATP when 30 combined suggested that the addition of AL to MAP(acronym: MAPAL) might safely reduce the average tumor ATP levels still lower than the average 15% of normal induced by MAP. We have not the funding opportunity to compare in the same tumors the

average % of induced ATP depletion by MAPA [REDACTED] as compared to MAP. However, the evaluation of MAPAL + an antitumor agent to MAP+ the same anticancer agent against advanced mammary tumors in mice showed MAPAL to be therapeutically more effective than MAP, and with no mortality and only an 8% weight loss (unpublished studies).

5 MAPAL, therefore, was the ATP-depleting regimen chosen to be evaluated against NCI-AR-RES (Adriamycin-resistant) 10 xenografts, and the therapeutic results are summarized in Table 2.

15 TABLE 2. THE ADDITION OF AN ATP-DEPLETING REGIMAN, MAPAL, ENABLES LO-DOSE ADRIAMYCIN TO KILL DRUG-RESISTANT CANCER CELLS IN ADRIAMYCIN-RESISTANT HUMAN MAMMARY CARCINOMA XENOGRAFTS BY THE NECROSIS CELL DEATH PATHWAY

Treatment Group ^a	n ^c	Body change ^c %	Wt	PR ^c %	p-Value ^d
1. Control	20	+6.6		0	
2. Adria ₁₀	57	-7.8		0	
3. MAPAL ^b	76	-3.8		18 (14/76)	0.0003
4. MAPAL → 2 ½ hr → Adria ₅	77	-7.9		48 (37/77)	<.0001
5. MAPAL+Adria ₅ (SIMULTANEOUSLY)	52	-9		46 (24/52)	< .0001

^a

20 Pooled experiments (99,103,108,112,114,115,116,117): Nude mice (nu/nu) with NCI-AR-RES mammary carcinoma xenografts averaged 100mg when treatment initiated. Indicated treatment administered in three cycles with a 14 day interval between cycles.

25 ^b MAPAL = 6-methylmercaptopurine riboside (MMPR₁₅₀) + 6-aminonicotinamide (6-AN₆) + PALA₁₀₀+ alanosine (AL₂₅₀); subscript

= mg/kg. PALA 1 hrs prior to (MMPR+6-AN+AL) 5 hrs prior to Adria. All agents i.p., except Adria i.v.

c Therapeutic observations recorded one week after the third course (35 days after initiating therapy.)

5 . PR= ≥ 50% decrease in tumor volume

d Compared to Adria Group 2. Comparing Group 3 VS Group 4 = <.0001; VS Group 5, 0.0011

Comment-- The combination of MAPAL and Adria (48% PR or 46% PR) is more effective in killing cells than just MAPAL (18% PR), and the use of only half- MTD of Adria in the presence of MAPAL (48%PR or 46% PR) is remarkably more effective in killing drug-resistant cancer than the full dose(MTD) of Adria (0% PR). The striking positive therapeutic results--almost a 50% P.R. rate using a 50% lower dose of Adria (with MAPAL) compared to a 0% P.R. rate with high dose Adria(alone) --auger well for an amelioration of the toxic side effects of high dose Adria in clinical trial along with greater therapeutic results.

20 It is stressed that there is no difference between the PR rates of Group 4, MAPAL 2½ hours prior to Adria, vs. Group 5, MAPAL simultaneously administered with Adria; namely, 48% P.R. vs. 46% P.R., respectively. It is therefore clear that the time differential (2½ hrs.) did not affect the results; 25 suspicion had been raised that the ATP-dependent p-glycoprotein drug efflux pump might be adversely affected by prior(2 ½ hr) ATP reduction in Group 4 as the reason for previously published positive therapeutic results with that time interval.

30 Molecular biology studies (Table 1) demonstrate that the necrosis cell death pathway can fully function (i.e., kill) to bypass a blocked apoptosis pathway, or can be fully evoked

following manipulation of energy metabolism to kill cells. The findings in these studies in non-drug resistant cells appear relevant to drug-resistant cells, which are blocked in apoptosis but not known to have blocks per se in the necrosis cell death pathway. Thus, if their apoptosis pathway is blocked but their necrosis pathway is "open", a combination of anticancer agents and ATP-depletion agents should be able to lower ATP to cytotoxic levels and selectively kill drug-resistant cells. The findings in these fifteen referenced studies imply that the necrosis cell death pathway could be the therapeutic opportunity to overcome drug-resistant cancer cells.

The primary similarity in the treated cancer cells of these molecular biology studies to drug-resistant cells is that apoptosis activity is prevented in both. In the drug-resistant cells triggered apoptosis cannot proceed to completion because of inherent **multiple** drug resistance factors; e.g., endogenous caspase inhibitors (so-called IAPs), genetic deletions of caspases, over-expressed anti-apoptotic bcl-2, mutations in p53 and other processing units in the apoptotic program, or in some individual cancer cells the presence of all of these genetically-induced resistance factors. Thus, apoptosis may be triggered (initiated), but not completed, by drug-treatment in the many above-noted molecular biology studies as well as in the drug-resistant cancer cells.

However, there is a significant difference in comparing necrosis pathways. In the molecular biology studies with **non**-drug resistant cells there are no resistance factors (i.e., obstacles) leading to the necrosis pathway, and the usual apoptosis cell death mode is readily "switched" to necrosis. In contrast, drug-resistant cells can have resistance factors

(e.g., drug efflux enzymes) that only allow for low drug concentrations that effect only partial completion of the necrosis pathway). High intracellular drug concentrations are required for cell death by necrosis. Necrosis will not occur unless the drug-target "hit" (usually to DNA) is of sufficient intensity to strongly activate PARP so as to result in a rapid and severe depletion of ATP to cell-killing levels ($\leq 15\%$ of normal). In drug-resistant cancer cells, the drug-induced necrosis pathway is initiated but does not proceed to completion (i.e., to death) because the drug-resistance factors of over-expressed drug efflux pumps (e.g., p-glycoproteins), and/or intracellular drug detoxifiers (e.g., glutathione), lower the intracellular drug level and diminish the drug-target interaction. Hence, although the result is still reduction of ATP, it is not low enough to be lethal-inducing (i.e., $\leq 15\%$ normal ATP). The necrosis pathway is only partially activated, and the ATP depletion does not fall to levels sufficient for cell-killing. Reductions of ATP to above the cell-killing threshold will only cause transient inhibition of cancer cell proliferation followed by complete recovery (7). Thus, following present chemotherapy, drug-resistant cancer cells are only sub-lethally-injured, recover, and grow back to kill the patient.

The molecular biology references (Table 1) suggest that a substantial but non-lethal degree of ATP depletion always occurs in anticancer agent-induced sub-lethally-injured drug-resistant cancer cells because there is only initiation, but not completion (i.e., to death) of the necrosis cell death pathway. Thus, since most effective DNA-damaging anticancer agents are themselves ATP-reducing agents, the further reduction of ATP to the ATP lethality threshold by an ATP-depleting regimen should kill these drug-resistant cells ---

and does (Table 1). Previous preclinical studies successfully overcoming one or another of the many drug-resistant factors have foundered in the clinical arena because drug-resistant factors are multifactorial. However, the novel ATP-depleting strategy is not aimed at any of the drug-resistant factors, but bypasses them all by directly killing drug-resistant cancer cells through their vulnerable necrosis cell death pathway.

10 PALM-Bromopyruvate (BrPA)

In non-drug-resistant tumors, Geschwind et al (18) have reported that a potent ATP-depleting agent (3-bromopyruvic acid, or BrPA), when delivered by "direct" intraarterial administration to liver-implanted rabbit tumors, eradicates nearly all viable cancer cells by necrosis, and also without harm to normal cells or to the animals. The absence of mortality and serious toxicity further obviates the notion that severe intra-tumor ATP depletion must be toxic to both 20 normal and cancer cells.

No ATP measurements were reported in the successful cancer cell-killing therapy with BrPA as a single agent. The very high concentration of BrPA achieved by the "direct" arterial delivery apparently depleted ATP in the "directly"- treated cancer cells to cell-killing ATP levels because the same dose of BrPA by systemic i.v. administration only inhibited tumor growth, apparently due to a diluted concentration by the whole blood volume only lowering ATP to levels above the cell-killing ATP threshold, levels shown to effect only inhibition of tumor growth. The level of ATP depletion is all important as regards the therapeutic result (ie, death of cells vs. their transient inhibition). This understanding provides

insight for the past failures and present success of ATP-depletion.

5 Previous attempts at ATP-depletion as therapy have failed due to the lack of understanding of how it would be effective in killing cancer cells. Only recently has there been data establishing that there is a cell-killing ATP threshold of 15% of basal levels and below(7-8), and that attaining this severe 10 lowering of intracellular ATP levels in cancer cells normally requires the concomitant administration of multiple ATP-depleting agents(1).

15 In line with our planned step by step development of a "best" multiple-ATP-depleting regimen, the incorporation of "selective" antimitochondrial agents (e.g., F16; 19) had been programmed for eventual inclusion in the ongoing development of the new and promising ATP-depleting therapeutic strategy. 20 Although BrPA has not been reported as "selective", it was noteworthy in reducing cellular ATP production via the inhibition of both glycolysis and oxidative phosphorylation (18). It was therefore substituted for the glycolysis inhibitor, 6-AN, in MAPAL. Without 6-AN, MAPAL includes only 25 PALA+Alanosine (AL) + MMPR (acronym: PALM). PALM+BrPA, or PALM-BrPA, inhibits adenine biosynthesis, glycolytic and mitochondrial ATP production. PALM-BrPA + Adri₅ vs MAPAL + Adri₅ were compared in therapeutic studies in multi-drug-resistant NCI-AR-RES (Adriamycin-resistant) human mammary tumor xenografts. Table 3 pools the data of four experiments.

30 TABLE 3. PALM-BRPA IS SIGNIFICANTLY SUPERIOR TO MAPAL IN ENHANCING ADRIAMYCIN-DRUG-RESISTANT CANCER CELL KILL. PALM-BRPA INDUCES SEVERE ATP REDUCTION (BY INHIBITING ADENINE BIOSYNTHESIS, GLYCOLYTIC AND MITOCHONDRIAL ATP PRODUCTION) THAT ENABLES LO-DOSE ADRIAMYCIN TO KILL ADRIAMYCIN-RESISTANT

CELLS IN HUMAN MAMMARY XENOGRAFTS BY THE NODALOSIS CELL DEATH PATHWAY.

Treatment Group ^a	n ^c	Body change ^c %	Deaths ^c %	PR ^c %	p-Value
1. Control	8	+5		0	
2. Adria ₁₀	15	-6		0	
3. MAPAL + Adria ₅	41	-8	4	34	
4. PALM + BrPA ₁₀ + Adria ₅	42	-13	0	62	^d 0.01*

5 a. Four pooled experiments (116, 117, 121, 122): Nude female mice with NCI-AR-Res human mammary carcinoma xenografts averaging 100mg when treatment initiated. Indicated treatments were administered in 3 cycles with a 14 day interval between cycles.

10 b. MAPAL = MMPr₁₅₀ + 6-AN₆ + PALA₁₀₀ + AL₂₅₀. PALM = PALA₁₀₀ + AL₂₅₀ + MMPr₁₅₀. BrPA = 3-Bromopyruvate₁₀. All agents i.p., except BrPA and Adria. i.v. Subscripts = mg/kg.

15 c. Therapeutic observation one week after the third course (35 days after initiating therapy). PR = $\geq 50\%$ reduction in tumor size compared to tumor size when treatment initiated. PR % = number PR per group \div surviving animals per group $\times 100$.

20 d. Compared to Group 3, MAPAL + Adria₅.
Comment-As previously (Table 2), the MTD of Adria, 10mg/kg, on this Adriamycin-resistant tumor is without tumor-regressing effect (0% PR), whereas Adria. at $\frac{1}{2}$ MTD in the presence of MAPAL causes an impressive number of partial tumor regressions in drug-resistant cells (34% PR), and PALM-BrPA + Adria ($\frac{1}{2}$ MTD) is remarkably much more effective (62% PR). With only these four experiments completed, the PALM-BrPA data is considered

preliminary. Nevertheless, PALM-BrPA+Adria₅ appears to have doubled (62%) the PR rate over MAPAL+Adria (34%). We have not had the funding opportunity to compare the degree of ATP depletion. Both combinations inhibit adenine supplies (i.e., by MMPR), both inhibit adenosine monophosphate biosynthesis (i.e., by AL), and both are glycolytic inhibitors, although by different mechanisms---PALM-BrPA by BrPA, and MAPAL by 6-AN. Although one may be a slightly stronger glycolytic inhibitor, the major difference clearly is that only PALM-BrPA is also a mitochondrial ATP inhibitor. Therefore, it could be anticipated that PALM-BrPA would be the stronger ATP depleter, and the therapeutic results confirm this expectation.

MAP with **two** ATP depleters effected a tumor ATP average to the cytocidal ATP threshold of 15% of normal (1). MAPAL with **three** ATP depleters apparently further reduced the average ATP level below this critical cytocidal ATP threshold level. PALM-BrPA with **four** ATP depleters apparently reduced this critical ATP average still more. Thus, with each incremental decrease in ATP depletion below the cytocidal threshold of 15% of normal, more drug-resistant cancer cells are killed.

Combining a number of ATP-depleting agents sometimes raises concern for anticipated toxic side-effects in normal tissues. However, cancer cells are apparently preferentially vulnerable to ATP depletion, and note that the markedly increased tumor-regressing results of PALM-BrPA, which combines four ATP-depleting components, is not accompanied by gross damage to normal tissues, or mortality.

30

OTHER ATP DEPLETERS THAT COULD CONTRIBUTE TO AN ATP-DEPLETING REGIMEN

A. Inhibitors of Mitochondrial ATP - There are a number of mitochondrial toxic small molecules that selectively accumulate in the mitochondria of tumor cells, compromise their functional integrity, have antiproliferative activity, and can cause a significant depletion of ATP pools. F16 is a small molecule that belongs to this group of compounds that have cancer cell mitochondria as a selective target (19). Although only tumor growth inhibitory as a single agent in vivo, its selectivity and low toxicity suggests that it could make a substantial contribution to ATP depletion as a component of a necrosis-inducing ATP-depleting combination (e.g., MAPAL-F16). F16's selectivity appears restricted to breast cancers. There are other such "selective" mitochondrial toxic molecules that display greater diversity in accumulating in the mitochondria of other types of cancer (20-21), and these should be evaluated in a multi ATP-depleting combination.

B. Inhibitors of Methylthioadenosine phosphorylase (MTAP) - MTAP is an important intracellular salvage enzyme for adenine (and methionine). Cancer cells that are deficient in MTAP are not able to salvage adenine (and methionine), and are markedly sensitive to inhibitors of the de novo synthesis of adenine nucleotides, especially alanosine which blocks de novo AMP synthesis (22). An inhibitor of MTAP would likely be a useful component of a multi-ATP-depleting combination, because MTAP plays a critical role in recycling adenine moieties from S-adenosylmethionine, derived originally from ATP, back to adenine nucleotide pools (23).

C. Inhibitors of De Novo Purine Synthesis Other Than 6-Methylmercaptopurine riboside (MMPR) - all tumors contain genetic instability and alterations, including chromosome losses (i.e., gene deletions) and gains (i.e., overexpressed

genes). (24) The genetic instability is reflected in the heterogeneity seen within individual tumors and among tumor of the same type.

5 Against this background, it is not surprising that MMPPR, which requires metabiotic activation by phosphorylation via adenosine kinase (25), meets with resistance in cancers devoid of adenosine kinase (26-27). Since MMPPR is an important component of MAPAL and PALM, the ATP-depleting strategy would 10 not be effective in adenosine kinase-deficient cancer cells. Also, cancer cells with overexpressed multidrug resistance protein 4, a drug-efflux pump that transports MMPPR out of the cell, would reduce the MMPPR intracellular concentration, and be a resistance mechanism (28). Substitution of MMPPR in the 15 multi-ATP-depleting regimen by antifolates that target purine synthesis should be as effective as MMPPR, might even be better, and should be evaluated. These novel antifolates include DDATHF (29), Agouron compound AG 2034 (30), and PDX (31-32).

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20

ATP-DEPLETING COMBINATION

(i.e., the ATP-depleting combination that, when co-administered with an anticancer agent effective against the target cancer, achieves cell-killing levels of ATP in drug-resistant cancer cells with the least toxicity to white blood cells and nerves.)

1. The initially employed ATP-depleting agents --6methyl-mercaptopurine riboside (MMPR)+6-aminonicotinamide (6-AN) -- were administered in combination with N-(phosphonacetyl)-L-aspartic acid (PALA), and received the acronym: MAP. MAP enhanced anticancer agent activity 30 against drug-resistant cancer cells.

2. Subsequently, MMPR and 6-AN were combined with alanosine (AL), and received the acronym:MAPAL (i.e., MMPR+6-AN+PALA+AL). MAPAL proved a more effective ATP-depleting agent than MAP. In *in vivo* studies in tumor-bearing animals, MAPAL, when co-administered with anticancer agents, evidenced greater anticancer activity than MAP, but with occasional evidence of greater myelotoxicity. MAPAL, nevertheless, is better than MAP.

10 3. The combination of PALA+AL+MMPR without 6-AN (acronym: PALM), when co-administered with anticancer agents, appears to produce the same or better antitumor activity than MAPAL without this occasional toxicity. MAPAL needs to be compared with PALM under the same conditions.

15 4. 6-AN inhibits glycolysis (1), a desirable ATP-depleting effect since the vast majority of solid cancers depend on glycolysis as a source of ATP (2). 6-AN acts by inhibiting the oxidative portion of the Pentose Phosphate Pathway (PPP) via inhibition of the second enzyme of the PPP. DHEA (dehydroepiandrosterone) inhibits the first enzymes of the oxidized version of the PPP, and, although inferior as an inhibitor of glycolysis to 6-AN is less toxic than 6-AN. But since the glycolytic production of ATP receives substrate contributions (fructose-6-phosphate and glyceraldehyde-3-phosphate) from the PPP, the oxidative portion of the PPP, DHEA will likely produce the same inhibition of glycolysis when administered in combination with an inhibitor of the non-oxidative portion of the PPP, oxythiamine (OT). Thus, PALM+DHEA+OT; acronym: PALMDOT.

Nevertheless, a greater blockade of the PPP might be accomplished by inhibiting both the first and the second

enzymes of the oxidative portion of PPP with DHEA+6-AN administration together with simultaneous inhibition of the non-oxidative portion of the PPP by OT; acronym: MAPALDOT. (If MAPALDOT is myelosuppressive, it may be made safe by a co-administration of G-CSF while affecting greater ATP depletion than PALMDOT.) PALMDOT and MAPALDOT need comparison.

5
10 EVALUATION OF THE DIFFERENCES BETWEEN ATP-DEPLETING REGIMENS
of MAPAL, PALM, PALMDOT AND MAPALDOT

15
20
25
30
35 Tumor Differences - Different tumor types (e.g., breast cancer vs. ovarian cancer vs. pancreatic cancer) may differ materially in the quantitative contribution of ATP they receive from different ATP-producing metabolic pathways (e.g., from glycolysis as compared to *de novo* purine metabolism). If the major contribution is, for example, from *de novo* purine synthesis, then it may be best to employ PALM as the ATP-depleting regimen, for PALM has two inhibitors of *de novo* purine synthesis, AL (alanosine) and MMPr (6-methyl-mercaptopurine riboside), and no antiglycolysis component. However, while there are exceptions, the vast majority of solid cancers depend on glucose through glycolysis as an energy source, and it would be imprudent to neglect this information. Hence, there should be evaluation of MAPAL, which contains not only MMPr and AL, but also has 6-AN, a proven inhibitor of glycolysis via the oxidative portion of the Pentose Phosphate pathway (PPP). But 6-AN may have toxicities that are non-pertinent to the goal of ATP depletion (such as rare neuroparalysis) and, therefore, it may be best to use DHEA, a less toxic inhibitor of the oxidative PPP. DHEA may be inferior to 6-AN as an inhibitor of glycolytic ATP production, but DHEA and 6-AN have not been previously compared in regard to reduction of glycolytic ATP, and the combination of DHEA + an inhibitor for the non-oxidative

portion of the [REDACTED], OT (oxythiamine), has not been evaluated (in combination) as regards inhibition of glycolytic ATP production.

5 Greater understanding of the above potential interrelationships may be obtained by comparative studies on three different cancers (the human breast cancer xenograft resistant to Adriamycin, the NCI/Adr-Res; the cisplatin (DDP) -doxorubicin-resistant human ovarian cancer xenograft, the 10 SKOV-3; and the S2 (TXT), a taxotere (TXT)-resistant human pancreatic cancer xenograft) as follows:

- 15 1. Controls
2. Cisplatin (DDP) MTD
3. Doxorubicin (DOX) MTD
4. Taxotere (TXT) MTD
5. MAP
6. MAPAL
7. PALM
8. PALMDOT
- 20 9. MAPALDOT
10. DDP half-MTD
11. DOX half-MTD
12. TXT half-MTD
- 25 13. MAP+DDP half-MTD; +DOX half-MTD; TXT half-MTD
14. MAPAL+ DDP half-MTD; +DOX half-MTD; +TXT half-MTD
15. PALM+ DDP half-MTD; DOX half-MTD; +TXT half-MTD
16. PALMDOT+ DDP half-MTD; +DOX half-MTD; +TXT half-MTD
- 30 17. MAPALDOT+DDP half-MTD;+ DOX half-MTD;+ TXT half-MTD

(The above 27 groups (10 tumor-bearing animals per group) cannot, practically-speaking, be included in a single 35 transplant experiment, but can be judiciously covered in a number of smaller experiments.)

40 Myelotoxicity Differences-- None of the above three anticancer agents alone at half-MTD, nor the various ATP-depleting regimens alone, cause myelosuppression in their tumor-bearing mice, but in combination (as in groups 13-17)

occasionally do [redacted] including a rare fatality [redacted] which can be prevented by either pyruvate administration, or omission of 6-AN, as in PALM. The indirect evidence, (myelosuppression only in combination, prevention by omission of 6-AN, or administration of pyruvate) suggests an adverse effect to a metabolic step of the glycolysis pathway in either the progenitor bone marrow cells or the peripheral blood leucocytes (PBL). Therefore, before treatment, and at intervals (24, 48, 72, and 96 hours) after treatment, bone marrow and PBL will be analyzed for changes in various steps of glycolysis and the PPP. An understanding of the changes may make it possible to identify a biomarker in glycolysis that will predict which tumor-bearing animal (i.e., patient) will undergo severe neutropenia and make possible early G-CSF administration for prevention as opposed to treatment by G-CSF. Such a biomarker in PBL might be glucose-6-phosphate dehydrogenase, known to be important for cell growth and in cell death(3-4).

The elaborate analysis of "best" ATP-depleting regimens, and control of PBL toxicity, detailed above is warranted by the importance of the drug resistance problem. Combination chemotherapy has been proven to cure the heterogeneity of a few types of cancer (e.g., testicular cancer), so there is clear evidence that it is possible to cure cancer with chemotherapy. But chemotherapy fails to cure most solid cancers because of drug resistance. Since there is strong scientific evidence in support of the ATP-depleting therapeutic strategy to circumvent drug resistance, the elaborate evaluation as planned above is because a careful application in the clinic of preclinical guidelines should enable clinical validation of the preclinically-proven strategy. Success is important, for the circumvention of drug

resistance factor will clear the way for therapeutic cure of many cancers.

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25 AN ATP-DEPLETING REGIMENT (e.g., MAPAL) + AN ANTICANCER AGENT (e.g., Taxotere) INDUCES PERMANENT GROWTH ARREST AND A SENESCENCE-LIKE PHENOTYPE IN ADVANCED HUMAN BREAST CANCER XENOGRAFTS (MDA-MB-468) THAT RESULTS IN CURE.

30 The induction of cell senescence, along with apoptosis, and other types of cell death (e.g., necrosis, mitotic catastrophe), can be a major response of cancer cells to cytotoxic and cytostatic agents. Thus, treatment conversion of cancer cells to senescent permanent proliferation arrest and subsequent cell death can add an important determinant of effective treatment outcome.

Neither the anti-cancer agent at MTD, nor the anticancer agent at half-MTD alone, nor the ATP-depleting regimen alone, effected senescence in the breast tumor xenografts. Therefore, this identification that only the combination of the ATP-depleting regimen with a moderate dose of anticancer agent can induce or facilitate senescence in tumor cells is an important finding for the improvement of cancer therapy.

PRESENT STATUS OF CANCER CHEMOTHERAPY AND DRUG RESISTANCE

Chemotherapy is the primary treatment once cancer becomes systemic (i.e., metastasizes). Most anticancer agents damage DNA in their target cancer cells, but the post-damage responses of apoptosis, necrosis, mitotic catastrophe and senescence are thwarted in drug-resistant cancer cells. The drug resistance factors are multiple, and range from mechanisms that limit the drug-target interaction (e.g., overexpression of drug efflux pumps, as p-glycoprotein, and intracellular detoxifiers, as glutathione) to genetic disruption of the apoptotic and senescence pathways. Drug-induced senescence, although difficult to induce, is increasingly being considered an important consequence of effective treatment(2,3). Mitotic catastrophe is a form of programmed cell death when the DNA-damaged cells exit a cell cycle arrest and undergo fatal endomitosis(1). It is the contribution of apoptosis to therapy-induced cell death (the "apoptosis concept" that is considered by most as the pivotal response program in drug-treated tumor cells (4). And necrosis, although occasionally listed as a drug-induced response, is usually not considered.

Yet, necrosis is the cell death pathway simultaneously initiated with apoptosis by drug-induced DNA damage that, unlike apoptosis, can be restored to the completion of cell death (5). Necrosis is due to severe ATP depletion (15% of

normal and below, a cell-killing level is prevented from attainment by drug-resistant factors (5). However, ATP is nevertheless reduced due to the activation of PARP by anticancer agent-induced DNA damage (5). Thus, unlike "mitotic catastrophe" and the cell death mode of apoptosis, which are initiated but not completed in drug-resistant cancer cells, the necrosis pathway in drug-resistant cells is partially completed (5). The anticancer agent-induced DNA damage, even though lessened by drug-resistant factors, reduces ATP and thereby "chemosensitizes" the drug-resistant cancer cell for further reduction to cell-killing ATP levels by the co-administration of an appropriate ATP-depleting regimen (5). It does not matter whether one or multiple drug-resistant factors are involved in the limitation of the necrosis pathway because the treatment to circumvent the drug-resistant factors is aimed at bringing the product of their collective blocking action-- i.e., the reduced ATP level -- to cell-killing levels. The ability to kill drug-resistant cancer cells by an apoptotic independent mechanism-- i.e., necrosis -- has been preclinically demonstrated, and is receiving NCI support for validation of the ATP-depleting strategy by clinical trial.

As noted above, there is growing evidence that senescence of cancer cells can be induced following chemotherapy and can contribute to the success of chemotherapy (2,3). It is of great additional interest that the same ATP-depleting strategy that enhances cancer cell death by inducing necrosis, also can induce senescence in cancer cells *in vivo*. (The latter gratifying results require additional confirmation in other tumor models and with other anticancer agents.)

Chemotherapy has cured a few types of human tumors. However, many human advanced solid cancers respond poorly to

chemotherapy because of drug-resistant cells. The ATP-depleting strategy could circumvent the latter problem, and thereby create the opportunity for chemotherapeutic cure of the more common human malignancies. The belief, and general 5 consensus that chemotherapy kills by apoptosis, and therefore that cells resistant to apoptosis are resistant to drug therapy, neglects the long-established fact that chemotherapy also kills by necrosis (5-7), and overlooks the evidence that cell death by blocked apoptosis can be "switched" to necrosis 10 (7-9). Overcoming drug resistance is the most important obstacle to the success of chemotherapy in the cure of advanced human cancers.

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What is claimed

1. A composition comprising a combination of ATP-depleting agents at concentrations which deplete the ATP level to at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor or an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside.
2. A composition comprising an effective amount of a combination of ATP-depleting agents at concentrations which deplete the ATP level to at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor or an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside.
3. The composition of claim 1 or 2, wherein said composition produces a substantially better effect than a composition without at least one of the following ATP-depleting agents: a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor or an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside.
4. The composition of claim 1, 2 or 3, further comprising a pyrimidine-depleting agent or a pyrimidine antagonist.
5. The composition of claim 1, 2 or 3, further comprising an anticancer agent.
6. The composition of claim 5, wherein the anticancer agent

to which the cancer is sensitive.

7. The composition of claim 5 or 6, wherein the anticancer agent is at approximately half of the maximum tolerated dose.
8. The composition of claim 1-7, wherein the ATP-depleting agents is 6-methylmercaptopurine riboside (MMPR), 6-Aminonicotinamide (6-AN), alanosine (AL) or a combination thereof.
9. The composition of claim 8, further comprising N-(phosphonacetyl)-L-aspartic acid (PALA).
10. The composition of claim 9, further comprising 3-bromopyruvic acid.
11. The composition of claim 1-10 wherein the ATP-depleting agents is 6-methylmercaptopurine riboside (MMPR), alanosine (AL) or a combination thereof.
12. The composition of claim 11, further comprising N-(phosphonacetyl)-L-aspartic acid (PALA).
13. The composition of claim 11, further comprising dehydroepiandrosterone (DHEA).
14. The composition of claim 11, further comprising oxythiamine (OT).
15. The composition of claim 11, further comprising dehydroepiandrosterone (DHEA) and oxythiamine (OT).
16. The composition of claim 11, further comprising 6-Aminonicotinamide (6-AN).

17. The composition of claims 1-16, further comprising a cytokine.

18. The composition of claim 17, wherein the cytokine is G-
5 CSF.

19. A pharmaceutical composition comprising the composition of claim 1-18 and a pharmaceutically acceptable carrier.

10 20. A method for treating a cancer subject comprising administering to the subject a combination of ATP-depleting agents at concentrations which deplete the ATP level to at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor or an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside.

20 21. A method for treating a cancer subject comprising administering to the subject a combination of ATP-depleting agents at concentrations which deplete the ATP level to at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor or an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, wherein said composition produces a substantially better effect than a composition without at least one of the following ATP-depleting agents: a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor or an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside.

22. The method of claim 20 or 21, further comprising a pyrimidine-depleting agent.

5 23. The method of claim 20 or 21, further comprising an anticancer agent.

24. The method of claim 23, wherein the cancer is clinically sensitive to the employed anti-cancer agent.

10 25. The method of claim 23 or 24, wherein the anticancer agent is at approximately half of the maximum tolerated dose.

15 26. A method for induction of cancer cell death comprising contacting said cancer cell with a combination of ATP-depleting agents at concentrations which deplete the ATP level to at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor or an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside.

25 27. A method for induction of cancer cell death comprising contacting said cancer cell with a combination of ATP-depleting agents at concentrations which deplete the ATP level to at least 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor or an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside, wherein said composition produces a substantially better effect than a composition

without at least one of the following ATP-depleting agents: a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor and an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside.

5 28. The method of claim 26 or 27, further comprising a pyrimidine-depleting agent.

10 29. The method of claim 26 or 27, further comprising an anticancer agent.

30. The method of claim 29, wherein the cancer is clinically sensitive to the employed anticancer agent.

15 31. The method of claim 29 or 30, wherein the anticancer agent is at half of the maximum tolerated dose.

20 32. A method for treating a cancer subject, or for the induction of cancer cell death, comprising administering to the subject a combination of ATP-depleting agents, a pyrimidine antagonist, and anticancer agent to which the treated cancer is sensitive, at concentrations which together collectively deplete the ATP levels to at least 25 15% of normal in cancer cells wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor or an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside.

30 33. A method for treating a cancer subject, or for the induction of cancer cell death, comprising administering to the subject a combination of ATP-depleting agents, a pyrimidine antagonist, and anticancer agent to which the 35 treated cancer is sensitive, at concentrations which

together collectively deplete the ATP levels to at least 15% of normal in cancer cells, wherein at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor or an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside wherein and said composition produces a substantially better effect than a composition without at least one of the following ATP-depleting agents: a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor and an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside.

- 15 34. The method of claim 32 or 33, wherein the anticancer agent is half of the maximum tolerated dose.
35. The method of claim 20-34, wherein the ATP-depleting agent is 6-methylmercaptopurine riboside (MMPR), 6-Aminonicotinamide (6-AN), alanosine (AL) or a combination thereof.
- 20 36. The method of claim 35, further comprising N-(phosphonacetyl)-L-aspartic acid (PALA).
- 25 37. The method of claim 35, further comprising 3-bromopyruvic acid.
- 30 38. The method of claim 35 wherein the ATP-depleting is 6-methylmercaptopurine riboside (MMPR), alanosine (AL) or a combination thereof.
39. The method of claim 35 further comprising N-(phosphonacetyl)-L-aspartic acid (PALA).

40. The method of claim 35 further comprising dehydroepiandrosterone (DHEA).

41. The method of claim 35 further comprising oxythiamine 5 (OT).

42. The method of claim 35 further comprising dehydroepiandrosterone (DHEA) and oxythiamine (OT).

10 43. The method of claim 35 further comprising 6- Aminonicotinamide (6-AN).

44. The method of claim 20-43 further comprising a cytokine.

15 45. The method of claim 44, wherein the cytokine is G-CSF.

46. A method for treating drug-resistant cancer cells comprising contacting the said cancer with a combination of ATP-depleting agents and an anticancer agent.

20 47. The method of claim 46, wherein the dose of said anticancer agent is at approximately half of the maximal tolerated dose.

25 48. The method of claim 46, wherein the ATP level is depleted to at least 15% of normal in cancer cells and at least one of the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor or an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside.

30 49. The method of claim 46, wherein the ATP level is depleted to at least 15% of normal in cancer cells and at

least one [REDACTED] the ATP-depleting agents is a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor or an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside and said composition produces a substantially better effect than a composition without at least one of the ATP-depleting agents: a mitochondrial ATP-inhibitor, a glycolytic inhibitor, a methylthioadenosine phosphorylase inhibitor and an inhibitor of De Novo purine synthesis other than 6-Methylmercaptopurine riboside.

- 5 50. A method for induction of cancer cell death comprising contacting said cancer cell with an agent capable of inducing necrosis in cancer cells.
- 10 51. The method of claim 50, wherein the agent is an ATP-depleting agent.
- 15 52. The method of claim 50 further comprising a pyrimidine-depleting regimen.
- 20 53. The method of claim 50 further comprising an anticancer agent.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/18716

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.

US CL : 514/45, 269; 536/27.21; 546/310; 562/11, 571; 564/118

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/45, 269; 536/27.21; 546/310; 562/11, 571; 564/118

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

File CA Plus structure, chemical name and inventor name searches

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93/23014 A1 (Martin et al.) 25 November 1993 (25.11.93), see entire document.	1-6, 20-24, 26-30, 32-34, 46-53
X	STOLFI et al. Biochemical Modulation of Tumor Cell Energy: Regression of Advanced Spontaneous Murine Breast Tumors with a 5-Fluorouracil-containing Drug Combination," Cancer Research. 01 August 1992, Vol. 52, No. 15, pages 4074-4081, see entire document.	1-6, 20-24, 26-30, 32-34, 46-53

<input checked="" type="checkbox"/>	Further documents are listed in the continuation of Box C.	<input type="checkbox"/>	See patent family annex.
"	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
12 NOVEMBER 2003	25 NOV 2003
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>Lawrence E. Crane</i> LAWRENCE ERIC CRANE Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/18716

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COLOFIORE et al. Biochemical Modulation of Tumor Cell Energy IV. Evidence for the Contribution of Adenosine Triphosphate (ATP) Depletion to Chemotherapeutically Induced Tumor Regression. Biochemical Pharmacology, 27 November 1995, Vol. 50, No. 11, pages 1943-1948, see entire document.	1-6, 20-24, 26-30, 32-34, 46-53
X	MARTIN et al. Marked Enhancement in vivo of Paclitaxel's (Taxol's) Tumor-Regressing Activity by ATP-Depleting Modulation. Anti-Cancer Drugs. 1996, Vol. 7, pages 655-659, see entire document.	1-6, 20-24, 26-30, 32-34, 46-53
X	KOUTCHER et al. Radiation Enhancement by Biochemical Modulation and 5-Fluorouracil. International Journal of Radiation Oncology Biology Physics. 01 December 1997, Vol. 39, No. 5, pages 1145-1152, see entire document.	1-6, 20-24, 26-30, 32-34, 46-53
X	GESCHWIND et al. Novel Therapy for Liver Cancer: Direct Intraarterial Injection of a Potent Inhibitor of ATP Production. Cancer Research. 15 July 2002, Vol. 62, pages 3909-3913, see entire document.	1-6, 20-24, 26-30, 32-34, 46-53
X	NORD et al. Apoptosis Induced in Advanced CD8F1-Murine Mammary Tumors by the Combination of PALA, MMPR and 6AN Precedes Tumor Regression and Is Preceded by ATP Depletion. Cancer Chemotherapy and Pharmacology. 1997, Vol. 40, pages 376-384, see entire document.	1-6, 20-24, 26-30, 32-34, 46-53

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/18716

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):

A61K 31/70, 31/505; C07H 19/16; C07D 211/72; C07F 9/22; C07C 229/00; C07C 241/00

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US01/116

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/16716

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **Claims Nos.:**
because they relate to subject matter not required to be searched by this Authority, namely:

2. **Claims Nos.:**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. **Claims Nos.: 7-19, 25, 31, and 35-45**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/18716

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

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